Microvascular Research 97 (2015) 130–136

Contents lists available at ScienceDirect

Microvascular Research

journal homepage: www.elsevier.com/locate/ymvre

Trypanosoma cruzi antigens induce inflammatory angiogenesis in a mouse subcutaneous sponge model☆

Francisca Hildemagna Guedes-da-Silva [b], Deena Shrestha [b], Beatriz Cristina Salles [b], Vivian Paulino Figueiredo [b], Laís Roquete Lopes [b], Luiza Dias [b], Lucíola da Silva Barcelos [d], Sandra Moura [a,b], Silvia Passos de Andrade [d], Andre Talvani [a,b,c,*]

[a] Departamento de Ciências Biológicas, Universidade Federal de Ouro Preto, Ouro Preto, MG, Brazil
[b] Programa de Pós-Graduação em Ciências Biológicas/NUPEB, Universidade Federal de Ouro Preto, Ouro Preto, MG, Brazil
[c] Programa de Pós-Graduação em Saúde e Nutrição, Universidade Federal de Ouro Preto, Ouro Preto, MG, Brazil
[d] Departamento de Fisiologia, ICB, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil

ARTICLE INFO

Article history:
Accepted 17 October 2014
Available online 27 October 2014

Keywords:
Trypanosoma cruzi
Inflammatory angiogenesis
Sponge model
Vascular endothelial growth factor (VEGF)
Chemokines

ABSTRACT

Acute inflammation and angiogenesis are persistent features of several pathological conditions induced by biological agents leading to the resolution of local and systemic events. Glycoproteins derived from the protozoan *Trypanosoma cruzi* are suggested to mediate angiogenesis induced by inflammatory cells with still undescribed mechanisms. In this study, we investigated the effects of total antigen from trypomastigote forms of *T. cruzi* (Y strain), inoculated in sponges 24 h after implantation in mice, on angiogenesis, inflammatory cell pattern and endogenous production of inflammatory and angiogenic mediators on days 1, 4, 7 and 14 post-implant. There was an increase in hemoglobin content and in the number of blood vessels associated with *T. cruzi* antigen stimuli on the 14th day, assessed by the hemoglobin of the implants and by morphometric analysis. However, these antigens were not able to increase type I collagen content on the 14th day. Parasite antigens also induced high production of vascular endothelial growth factor (VEGF) and inflammatory mediators TNF-alpha, CCL2 and CCL5 on the 7th day in sponges when compared to the unstimulated group. Neutrophils and macrophages were determined by measuring myeloperoxidase (MPO) and N-acetyl-β-D-glucosaminidase (NAG) enzyme activities, respectively. Only NAG was increased after stimulation with antigens, starting from day 4 and peaking at day 7. Together, these data showed that antigens from the Y strain of *T. cruzi* are able to promote inflammatory neovascularization probably induced by macrophage-induced angiogenic mediators in *T. cruzi* antigen-stimulated sponges in Swiss mice.

Introduction

Trypanosoma cruzi is a flagellate protozoan capable to induce a persistent inflammatory response in mammalian hosts triggered by its membrane glycoprotein molecules that act on Toll like- and other primitive receptors in immune cells resulting in the releasing of inflammatory mediators (Campos et al., 2004, Talvani and Teixeira 2011; Gravina et al., 2013). This parasite infection can affect myocardial fibers and cardiac, esophagus and colon neurons culminating in loss of functionality and remodeling in the heart and in the digestive organs (Koberle, 1961; Prata, 2001). The infection-associated immunopathology and microvascular abnormalities are crucial aspects in the generation of heart disease which is characterized by fibrosis, myocytolysis, thromboembolism, dysrhythmia and cardiac hypertrophy (Rassi et al., 2009).

In particular, infection caused by parasites results in inflammation which in turn promotes angiogenesis which is defined as the formation of new capillaries by sprouting or by intussusception from pre-existing vessels (Folkman, 2006). The development of this vascular network is a tightly controlled mechanism performed by physiological and/or pathophysiological stimuli. In particular, in the microenvironment of injured tissue where there are low levels of oxygen as well as products of necrotic cells or a set of inflammatory mediators released by recruited cells, inflammation often contributes to generation of an angiogenesis designated as "inflammatory angiogenesis" (Kim et al., 2013). Macrophages/monocytes, due their plasticity in the immune response, use to play a pivotal role in this inflammatory angiogenesis restoring tissue homeostasis (Dirkx et al., 2006; Biswas et al., 2008; Ploeger et al., 2012). Inflammatory angiogenesis seems to be intimately involved in the reestablishment of injured tissue in chronic inflammatory disorders from

☆ Disclosures: Authors do not have conflict of interest regarding this publication.

* Corresponding author at:Universidade Federal de Ouro Preto, ICEB, Campus Morro do Cruzeiro, 35400-000 Ouro Preto, MG, Brazil. Fax: +55 31 3559 1680.
E-mail address: talvani@nupeb.ufop.br (A. Talvani).

http://dx.doi.org/10.1016/j.mvr.2014.10.007
0026-2862/

distinct etiopathogenesis such as autoimmune, metabolic or infectious diseases.

Upon infection, *T. cruzi* appears to translocate a protozoan-like calcium binding protein named calreticulin from the endoplasmic reticulum to the surface where this molecule exerts inhibition of some innate mechanisms of immunity favoring parasite surveillance and, in parallel, binds to endothelial cells inhibiting the angiogenesis process (Molina et al., 2005; López et al., 2010; Ramírez et al., 2012). However, few studies evaluating angiogenesis exist using live *T. cruzi* or parasite molecules in experimental animals. Mimicking a condition with absence of live parasites, we propose to characterize the sequential dynamics of inflammatory cell recruitment, newly formed blood vessels, chemokine production and fibrogenesis using subcutaneous polyester–polyurethane sponge implants in mice under stimuli with antigens extracted from trypomastigote forms of *T. cruzi* (Y strain).

Material and methods

Animals and sponge disc implantation

Ten week-old female Swiss mice were bred and maintained at Center of Animal Science (CCA) from the Universidade Federal de Ouro Preto, UFOP. After sponge implantation, animals were maintained in individual cages with food/water *ad libitum* and in heated shelves under temperature and humidity controls.

Polyester–polyurethane sponge discs of 5 mm thick and 8 mm diameter (Vitafoam Ltd, Manchester, UK) were used as the matrix for the fibrovascular tissue growth. Discs were sterilized by soaking overnight in 70% v/v ethanol and by boiling in distilled water for 15 min before the implantation surgery. Mice were anesthetized by intra-peritoneal injection of 4 μL g^{-1} of a mixture of ketamine (150 mg kg^{-1}) and xylazine (10 mg kg^{-1}), dorsal hair shaved and skin wiped with 70% of ethanol. Sponge discs were aseptically implanted into a subcutaneous pouch, previously made with a curved tweezer, through a 1-cm long dorsal midline incision. Post-operatively, the animals were monitored for discomfort, distress or any signs of infection at the implant site, assuring an uneventful return to normalcy in a clean, dry and warm environment and keeping them safe from injuries by other animals.

All procedures here were in accordance with the guidelines issued by the Brazilian College of Animal Experimentation (COBEA) and this proposed experiment was previously approved by the Ethics Committee in Animal Research at UFOP-CEUA (Protocol No.#043/2010).

Experimental design ofT. cruzi-antigen stimulation in subcutaneous sponge

To obtain total antigen from *T. cruzi*, fresh blood from Swiss mice containing 10^7 trypomastigote forms of the Y strain of this protozoan was added together with cultured Vero cells, that are derived from the kidney of an African green monkey and are one of the more commonly used mammalian continuous cell lines (Ammerman et al., 2008). These Vero cells were maintained at 37 °C in minimum essential medium (MEM) enriched with 5% fetal bovine serum (FBS) for 3 days. After this period, the supernatant containing trypomastigotes was removed from this culture by centrifugation at 120 ×*g*, at 26 °C for 3 min and parasites were washed three times with sterile PBS. Then, trypomastigote forms were counted, suspended in sterile phosphate buffer solution (PBS) and stored at −20 °C. The solution containing parasites was then harvested, centrifuged at 2800 ×*g* for 15 min at 4 °C and filtered through a membrane (pore size, 0.22 μm; Millipore, Bradford, MA). The supernatant antigens were obtained using an equivalence of 10^7 parasites per 0.2 mL of PBS and, then, stored at −20 °C to be used later.

Total antigen from *T. cruzi* (10^7/0.2 mL) or 0.2 mL of vehicle (PBS) was inoculated intrasponge in mice 24 h after the surgery of sponge implantation. The effects of the *T. cruzi* antigen on angiogenesis (hemoglobin content) and on inflammation (neutrophil, macrophage and fibrosis) and collagen content were assessed on days 1 (control), 4, 7 and 14 after sponge implantation. In addition, the pattern of angiogenesis and inflammation on the day of implantation (day 0) was also investigated. For each time-point of investigation, sponges from 10 mice were evaluated: 6 by biochemistry assays (hemoglobin, myeloperoxidase, N-acetylglucosaminidase and collagen) and 4 by morphometric analysis and blood vessel quantification, as described below.

Quantification of angiogenesis by hemoglobin measurement

Mice were euthanized by overdose of anesthesia and sponge implants were excised carefully, dissected from adherent tissue and weighed. Hemoglobin content was detected in the tissue using the Drabkin method (Drabkin and Austin, 1935). Each implant disc was homogenized (IKA Werk T-10 basic, Germany) in 2.0 mL Drabkin reagent (Labtest, São Paulo, Brazil) and centrifuged at 10,000 ×*g* for 15 min. The supernatants were filtered through a 0.22 μm filter (Millipore, Bedford, MA). Hemoglobin (Hb) analysis presents a good correlation with other methods for the detection and quantification of angiogenesis (Hu et al., 1995). Therefore, hemoglobin content in the samples was quantified colorimetrically at 540 nm in a spectrophotometer (Emax, Molecular Devices, Sunnyvale, CA) and its concentration calculated from a known amount of hemoglobin assessed in parallel. The data were expressed as μg Hb/mL/mg of wet sponge.

Quantification of neutrophil infiltration by myeloperoxidase (MPO) and macrophage infiltration by N-acetylglucosaminidase (NAG) activity measurement

The extent of neutrophil accumulation in the implants was measured by assaying myeloperoxidase (MPO) activity as previously described (Barcelos et al., 2004). After processing the supernatant of the implants for the hemoglobin assessment, a part of the corresponding pellet was weighed, homogenized in (2 mL) pH 4.7 buffer (0.1 M NaCl, 0.02 M Na_3PO_4, 0.015 M Na_2-EDTA) and centrifuged at 4 °C at 12,000 ×*g* for 10 min. The pellets were suspended in 0.05 M sodium phosphate buffer (pH 5.4) containing 0.5% hexa-1,6-*bis*-decyltrimethylammonium bromide (HTAB). MPO activity in the supernatant samples was assayed by measuring the change in absorbance (optical density — OD) at 450 nm using 3,3′-5,5′-tetramethylbenzidine (TMB) prepared in dimethyl sulfoxide (DMSO) in a final concentration of 1.6 mM and H_2O_2 (0.3 mM) in the sodium phosphate buffer, pH 6.0. The reaction was terminated by the addition of 50 μl of H_2SO_4 (4 M).

In parallel, the infiltration of mononuclear cells into the implants was quantified by measuring the levels of the lysosomal enzyme N-acetyl-β-D-glucosaminidase (NAG) which is present in high levels in activated monocytes/macrophages (Barcelos et al., 2004; Marques et al., 2011). Part of the pellet that remained after the hemoglobin measurement was kept for this assay. These pellets were weighed, homogenized in NaCl solution (0.9% w/v) containing 0.1% v/v Triton X-100 (Promega) and centrifuged (3000 ×*g*— 10 min at 4 °C). Samples of the resulting supernatant (100 μL) were incubated for 10 min with 100 μL*p*-nitrophenyl-N-acetyl-β-D-glucosaminide (Sigma) prepared in the citrate/sodium phosphate buffer (0.1 M citric acid, 0.1 M Na_2HPO_4— pH 4.5) to yield a final concentration of 2.24 mM. The reaction was stopped by the addition of 100 μL of 0.2 M glycine buffer (pH 10.6). Hydrolysis of the substrate was determined by measuring the absorption at 400 nm. MPO and NAG activities were expressed as the change in OD per milligram of wet sponge.

Quantification of collagen content in sponge discs

Total soluble collagen was measured in whole sponge homogenates by the Sirius Red reagent based-assay (Lima et al., 2014). Sponge discs from 4 animals per group were homogenized in 1 mL of PBS and 50 μL of the sample mixed with 50 μL of Sirius Red reagent. The collagen-

dye complex was centrifuged at 5000 ×g for 10 min and the pellet washed with 500 μL of ethanol (99% pure and methanol free). One milliliter of a 0.5 M NaOH solution was added to the remaining collagen-bound dye pellet. The color intensity of the samples was measured at 540 nm and the calibration curve set up on the basis of the gelatin standard (Merck). Results are expressed as μg collagen/mg wet sponge.

Immunoassays for CCL2, CCL5, TNF-alpha and VEGF

The homogenate of squeezed sponge obtained previously from MPO and NAG measurements, processed in 1.0 mL of PBS (pH 7.4) containing 0.05% Tween-20 (Difco) was once more followed by centrifugation at 4 °C for 10 min at 10,000 ×g and stored at 80 °C. Briefly, soluble cytokines TNF-alpha, CCL2/MCP-1, CCL5/RANTES and VEGF (PeproTech S.A., Mexico, DF) from sponge homogenates were evaluated in 96-microtiter plates coated with their specific murine monoclonal antibodies for 18 h at 4 °C and then washed with PBS containing 0.05% Tween-20 (wash buffer). Non-specific binding sites were blocked with 1% bovine serum albumin (BSA) in PBS and, 1 h later, plates were rinsed with the wash buffer and samples (diluted in 0.1% BSA in PBS) were added to the wells, followed by incubation for 4 h at 4 °C. Plates were then washed and an appropriate second horseradish peroxidase-conjugated polyclonal antibody against the cytokine was added to the wells. Plates were then washed and the chromogen substrate OPD N-phenylendiamine, Sigma) diluted in 0.03 M citrate buffer (pH 5.0) containing 0.02% 30 v/v H_2O_2 was added. The plates were incubated in the dark for 30 min at room temperature. The reaction was stopped with 2 N H_2SO4 solution (50 μL/well) and intensity of the color measured at 492 nm on a spectrophotometer (Emax-Molecular Devices). All samples were measured simultaneously in duplicate and results expressed as picograms of cytokine per milligram wet sponge.

Morphometric analysis and blood vessel quantification

To examine the degree of neovascularization in the implants stimulated or not with *T. cruzi* antigens, a total of 16 sponge discs (four discs for each time point — days 1, 4, 7 and 14 after implantation) were processed for histological assessment. The implants were embedded in paraplast (Erv-Plast — Erviegas Instrumental Cirurgico Ltda, São Paulo, Brazil) and sections of 5 mm were obtained and stained with hematoxylin and eosin (HE). Microscopic images of cross-sections (5 μm) were obtained with a planapochromatic objective 40× lens in light microscopy and a countable microvessel was defined as a structure with a lumen that contained red blood cells or not and with nucleated endothelium cells margining. New and old vessels as well as inflammatory leukocyte infiltration were scored by counting of a total of 74,931 μm^2 analyzed sponge section (Moura et al., 2009). Images were obtained through a Leica DM 5000 B microcamera (Leica Application Suite, model 2.4.0R1) and processed by the software Leica Qwin V3 image analyzer. All slides from sponges with or without *T. cruzi* antigens were reviewed in blind by one pathologist.

Statistical analysis

All results presented here are shown as the mean +/− SEM. Comparisons between two groups were carried out using Student's t-test for unpaired data. Three or more group comparisons were carried out using one-way ANOVA and differences between these groups were assessed by the Student–Newman–Keuls multiple comparisons test. Different letters in the graphics mean significant ($p < 0.05$) and equal letters mean absence of difference among groups.

Results

Leukocyte accumulation in the implant-induced angiogenesis

Myeloperoxidase is a neutrophil enzyme and it was found in high levels on the first to the fourth days post-implant (Fig. 1A) with similar patterns under *T. cruzi* antigen and vehicle (PBS) stimuli. Another enzyme N-acetyl-glucosaminidase, a representative of activated monocytes/macrophages into the inflammatory site, was found to be associated with the presence of the *T. cruzi* antigen in the sponge and evidenced on the 7th day (Fig. 1B). One week later (14th day), the monocyte level in the sponge with the antigen was still higher, as evidenced by the NAG enzyme activity, but was similar between both stimuli (vehicle and *T. cruzi* antigens). High levels of hemoglobin content in the sponge inoculated with Y strain *T. cruzi* antigens were detected on the 14th day (Fig. 1C). However, no difference in the collagen content on the 14th day may be due to the recent chronic phase for this specific sponge implant model (Fig. 1D). In addition, those animals inoculated with 0.2 mL of PBS intrasponge did not affect the angiogenesis profile when compared to non-injected sponges (data not shown).

Inflammatory and angiogenic mediators in the sponges

The inflammatory mediators and the vascular endothelial growth factor (VEGF) were measured and a high production of TNF-alpha (Fig. 2A), CCL2 (Fig. 2B), CCL5 (Fig. 2C) and VEGF (Fig. 2D) was detected on the 7th day in the sponge implant associated with the stimulation with *T. cruzi* antigens.

New vessels in the sponge disc matrix

Histological sections demonstrated a high number of new vessels on day 14th in association with the *T. cruzi* antigen stimulus (Fig. 3). In these histological sections, there were no infection and rejection signals in the sponge during all days of experiment. Besides, the sponges showed fibrovascular stromal growing in all 4, 7 and 14 days after subcutaneous implantation as illustrated in Fig. 4. In addition, increases of polymorphonuclear and mononuclear cells/fibroblasts and vascularization were also evident on days 4, 7 and 14, respectively, in those sponge implants stimulated with *T. cruzi* antigens (Y strain).

Collagen deposition was also evaluated in histological sections stained with picrosirius-red, demonstrating an initial predominance of both mature type I (Fig. 5A) and immature type III (Fig. 5B) collagens on the 4th day after implantation with *T. cruzi*-antigen stimuli. However, while type I collagen increased proportionately in the sponge matrix until the 14th day in the presence of vehicle, those animals that received the inoculum with the antigen of *T. cruzi* presented the production of this collagen to be stabilized (Fig. 5A).

Discussion

Angiogenesis, a well-defined physiological condition, has been frequently associated with inflammation in pathological disorders from distinct genesis, including parasite infection. The *T. cruzi*-induced inflammatory mediators amplify and promote a self-perpetuation of the systemic and local (e.g. heart) inflammation culminating in cellular destruction, local hypoxia, collagen deposition, fibrosis, remodeling and loss of functionality of the heart (Factor et al., 1985; Coura, 2007). Recruited leukocytes act as sources of inflammatory and angiogenic factors and there is a wellspring for the generation of new vessels in the cardiac tissue,even though there is an augment of chemokines (CCL2, CCL3, CCL5) and inflammatory cytokines (TNF-alpha, IFN-gamma, IL-12, IL-17) which in turn favors the production of the angiogenic factor (VEGF).

After *T. cruzi* invasion, the mammalian host experiences two stages of infection: (i) the initial phase where there is a high load of circulating

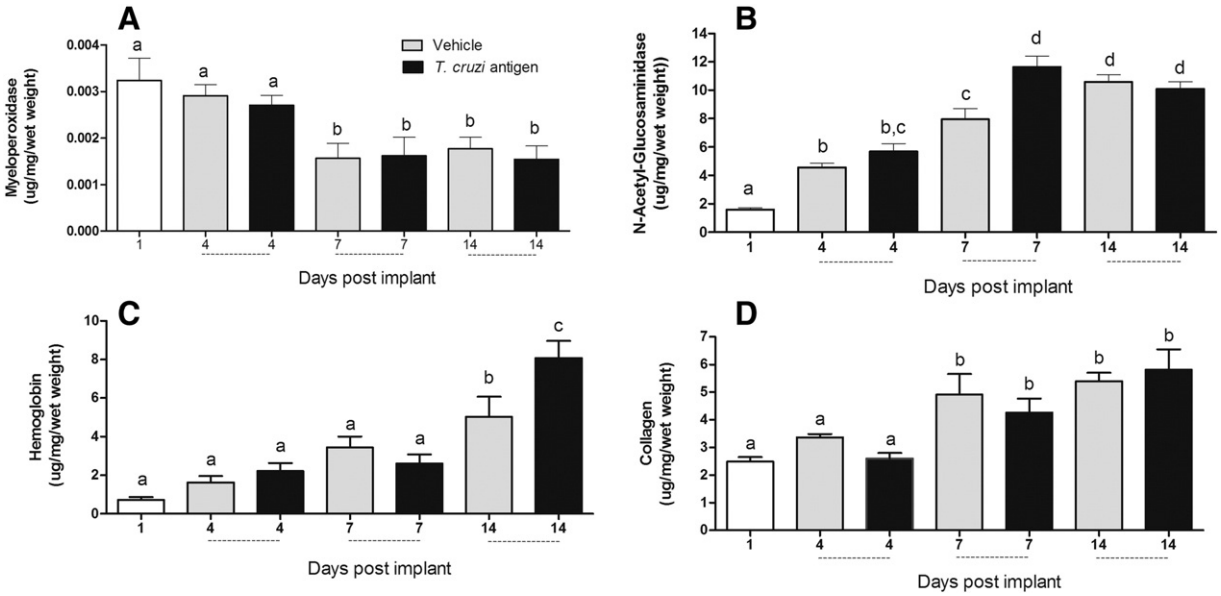

Fig. 1. Kinetic of inflammatory parameters in sponge matrix. Subcutaneous sponge implants stimulated with *T.cruzi* antigens or vehicle (PBS) were excised during 1, 4, 7 and 14 days to measure neutrophil enzyme myeloperoxidase (A), macrophage enzyme N-acetyl-glucosaminidase (B), hemoglobin (C) and collagen content (D) through biochemical analysis. Bars are representative of 6 sponge implants and different letters mean statistical difference with $p < 0.005$.

parasites as well in the muscle cells and (ii) a long-term phase where the persistence of parasites in tissues is more evident and the immune system remains on alert eliminating tissue parasites which persist releasing their antigens in the circulation. The effects of these antigens to the host system are still unclear. The subcutaneous sponge model, a well-established model for angiogenesis investigation was chosen due to the possibility to study the role of antigens of *T. cruzi* instead of live parasites. Studies involving the sponge model showed that this model presents an initial phase (1–3 days) with the predominance of neutrophils, an intermediate stage (4–8 days) with the predominance of macrophages and a final stage (8–15 days) where there is reduction of inflammatory cells with the predominance of blood vessels and collagen in the matrix of the sponge (Andrade et al., 1987; Barcelos et al., 2005; Teixeira et al., 2005; Marques et al., 2011). This is the first study involving administration of total antigen of the Y strain of *T. cruzi* and as demonstrated in Fig. 6, it can be summarized that *T. cruzi* was able to release high TNF-alpha and CCL2 and low VEGF in the four initial days post-implant, probably conducted by neutrophils. The apex of the inflammatory response was on the 7th day where high levels of TNF-alpha and CCL2 were maintained side by side with high levels of

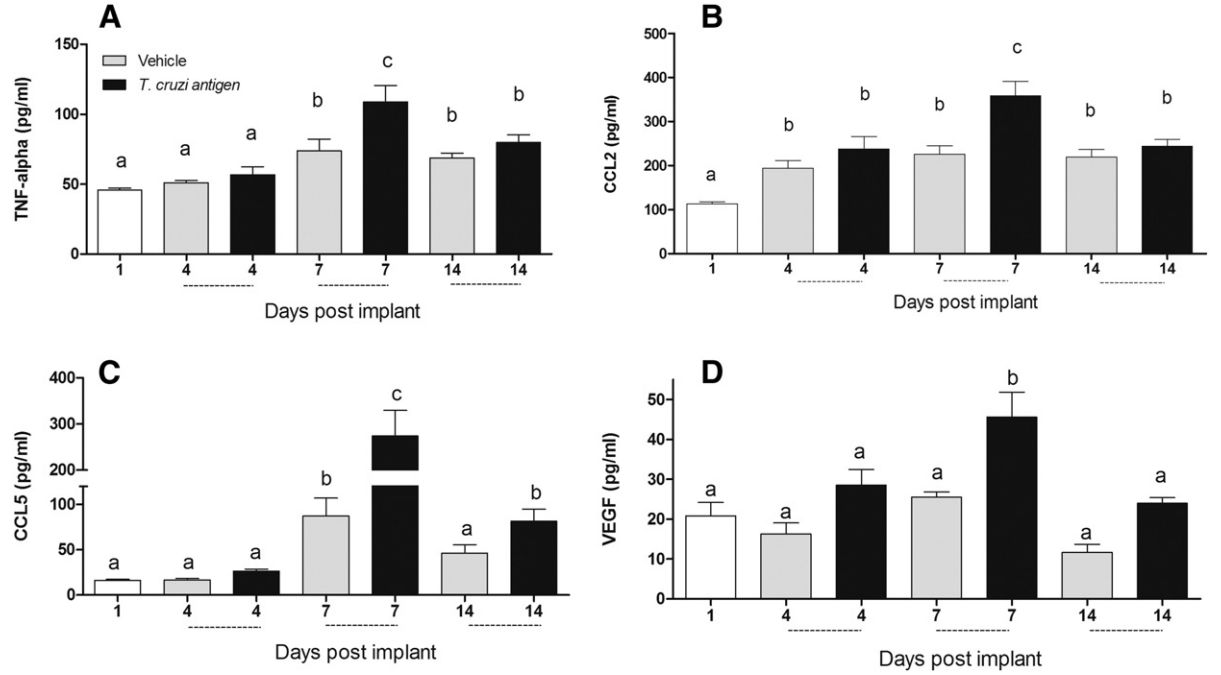

Fig. 2. Inflammatory and angiogenic mediators in sponge matrix. Subcutaneous sponge implants stimulated with *T. cruzi* antigens or vehicle (PBS) were excised during 1, 4, 7 and 14 days to measure TNF-alpha (A), macrophage chemoattractant protein/CCL2 (B), Regulated upon Activation, Normal T cell Expressed and Secreted/CCL5 (C) and vascular endothelial growth factor (D) through biochemical analysis. Bars are representative of 6 sponge implants and different letters mean statistical difference with $p < 0.005$.

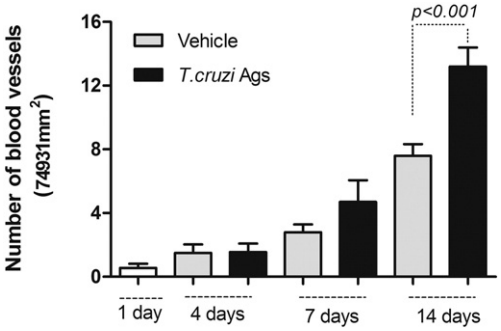

Fig. 3. Quantification of blood vessels. Subcutaneous sponge implants stimulated with *T. cruzi* antigens or vehicle (PBS) were excised during 1, 4, 7 and 14 days for histological preparation. A number of blood vessels were quantified in optical microscopy using morphological parameters (presence of red cells inside and the presence of nuclei of endothelial cells in the margin of the vessel). Statistical difference is represented by $p < 0.005$.

VEGF. This angiogenic factor was possibly responsible for the increasing of blood vessels as well as the inflammatory background for the type I collagen detected on the 14th day post-infection.

Modulation of the development of the vascular network in a *T. cruzi* infected tissue may constitute the strategy to ameliorate this pathological condition. Indeed, the role of angiogenesis in *in vivo* system associated with parasite infection is still controversy. The inflammation and pro-angiogenic factors are induced by antigens of *T. cruzi* and local hypoxia, respectively. During infection, the new vessels formed in *T. cruzi*-infected tissues act to nourish injured sites with increase blood nutrients and O_2 supply. However, an increase in the number of blood vessels also favors high leukocyte recruitment into the tissue to eliminate parasites as well as amplify the host tissue destruction. Even though our current results support an inflammatory angiogenesis, parasites exert an inhibition of angiogenesis through calreticulin release in *in vitro* and few *in vivo* studies (Ramírez et al., 2011, 2012). In this

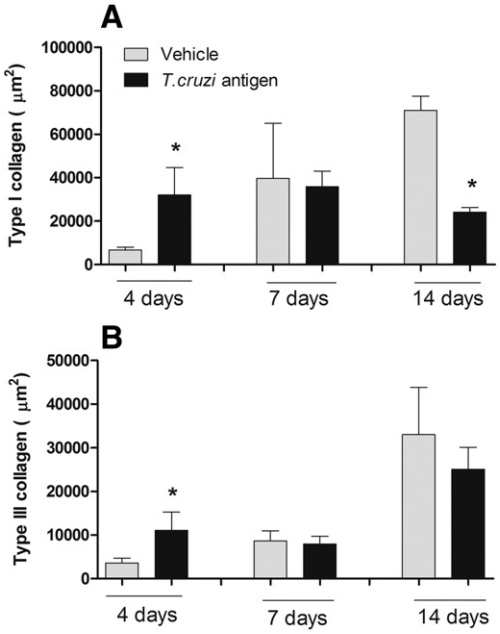

Fig. 5. Collagen in the sponge implant matrix. Histological sections were processed and sponge implants stained with picrosirius red for collagen type I (A) and collagen type III (B) assessment. * represents statistical difference between sponge implants stimulated with *T. cruzi* antigens and vehicle (PBS) in 4, 7, and 14 days post-implant.

regard, the use of pro- and anti-angiogenic therapies after experimental *T. cruzi* infection seems suitable and needs future elucidation in favor of mammalian host benefits.

In summary, our study demonstrates the inflammatory and pro-angiogenic effects after the administration of total antigens from the Y strain of *T. cruzi* in the subcutaneous model of sponge implants. In this

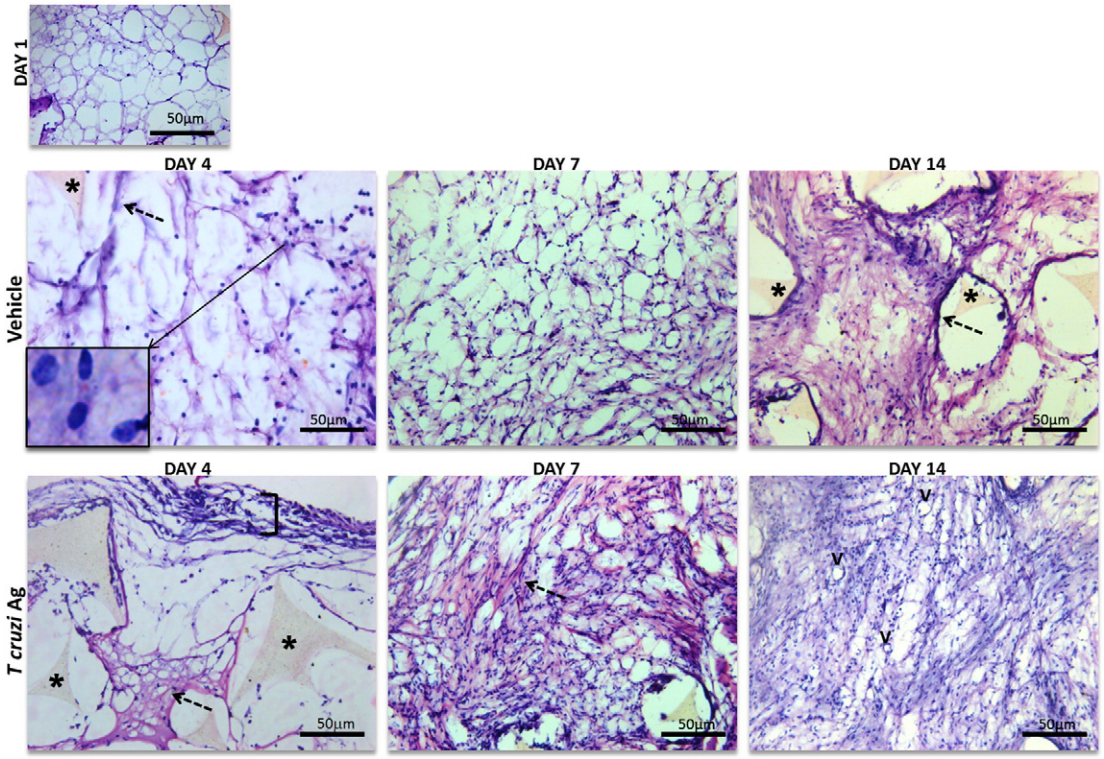

Fig. 4. Photomicroscopy of histological sections of sponge implants. Sections of 5 µm of subcutaneous sponge implants stimulated with *T.cruzi* antigens or vehicle (PBS) were stained in hematoxylin and eosin in days 1, 4, 7 and 14 post-implant. Sponge implants (*) were gradually filled out by inflammatory mononuclear cells (arrows) and by neovessels (v), fibroblasts and collagen fibers. In the box, macrophages are represented and (]) means the capsule of the connective tissue with the presence of inflammatory cells. Bar = 50 µm.

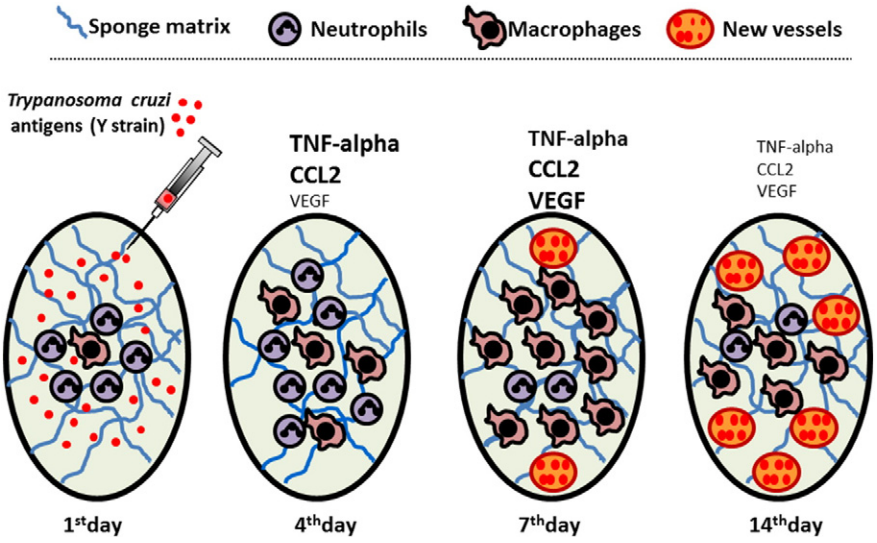

Fig. 6. Representative scheme of inflammatory and angiogenic parameters in subcutaneous sponge implants after stimulation with *T. cruzi* antigens. Increased word size means high production and reduced word size means low production of each soluble mediator.

study, the source of the antigens was the Y strain of *T.cruzi*, whose pathological characteristics are to induce myocarditis beginning on the 7th day post-infection in *Mus musculus* (Swiss mice) and to promote an extensive cardiac inflammation between the 12th and 22nd days post-infection, in particular in the left ventricle (Brener, 1973; de Diego et al., 1991). In light of immune response and tissue damage, our results propose a general idea of the free *T. cruzi* antigens during a long term experimental infection and their repercussion to the angiogenesis in the infected and/or inflamed tissues. Further studies involving different parasites with different genetic backgrounds are needed toward angiogenic therapy in experimental *T. cruzi* infection.

Conclusion

We conclude that antigens from the Y strain of *T. cruzi* are able to promote inflammatory neovascularization probably induced by macrophage-induced angiogenic mediators in *T. cruzi* antigen-stimulated sponges in Swiss mice.

Acknowledgments

This work was supported by grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq Processo#476229/2009-0), Fundacão de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG APQ-02642-10), International Society for Infectious Disease (ISID/EUA — Small Grant/2009) and Universidade Federal de Ouro Preto (UFOP). AT, LSB and SPA are recipients of productivity awards from CNPq.

References

Ammerman, N.C., Beir-Sexton, M., Azad, A.F., 2008. Growth and maintenance of Vero cell lines. Curr. Protoc. Microbiol. 11 (A.4E-1 – A.4E.7).
Andrade, S.P., Fan, T.P., Lewis, G.P., 1987. Quantitative *in-vivo* studies on angiogenesis in a rat sponge model. Br. J. Exp. Pathol. 68, 755–766.
Barcelos, L.S., Talvani, A., Teixeira, A.S., Cassali, G.D., Andrade, S.P., Teixeira, M.M., 2004. Production and *in vivo* effects of chemokines CXCL1-3/KC and CCL2/JE in a model of inflammatory angiogenesis in mice. Inflamm. Res. 53, 576–584.
Barcelos, L.S., Talvani, A., Teixeira, A.S., Vieira, L.Q., Cassali, G.D., Andrade, S.P., Teixeira, M.M., 2005. Impaired inflammatory angiogenesis, but not leukocyte influx, in mice lacking TNFR1. J. Leukoc. Biol. 78, 352–358.
Biswas, S.K., Sica, A., Lewis, C.E., 2008. Plasticity of macrophages function during tumor progression: regulation by distinct molecular mechanisms. J. Immunol. 180, 2011–2017.
Brener, Z., 1973. Biology of *Trypanosoma cruzi*. Annu. Rev. Microbiol. 27, 347–382.
Campos, M.A., Closel, M., Valente, E.P., Cardoso, J.E., Akira, S., Alvarez-Leite, J.I., Ropert, C., Gazzinelli, R.T., 2004. Impaired production of proinflammatory cytokines and host resistance to acute infection with *Trypanosoma cruzi* in mice lacking functional myeloid differentiation factor 88. J. Immunol. 172, 1711–1718.
Coura, J.R., 2007. Chagas disease: what is known and what is needed—a background article. Mem. Inst. Oswaldo Cruz 102 (Suppl. 1), 113–122.
de Diego, J.A., Penin, P., del Rey, J., Mayer, R., Gamallo, C., 1991. A comparative pathological study of three strains of *Trypanosoma cruzi* in an experimental model. Histol. Histopathol. 6, 199–206.
Dirkx, A.E., Oude-Egbrink, M.G., Wagstaff, J., Griffioen, A.W., 2006. Monocyte/macrophage infiltration in tumors: modulators of angiogenesis. J. Leukocyte Biol. 80, 1183–1196.
Drabkin, D.L., Austin, J.H., 1935. Preparations from washed blood cells; nitric oxide hemoglobin and sulphemoglobin. J. Biol. Chem. 112, 51–56.
Factor, S.M., Cho, S., Wittner, M., Tanowitz, H., 1985. Abnormalities of the coronary microcirculation in acute murine Chagas' disease. Am. J. Trop. Med. Hyg. 34, 246–253.
Folkman, J., 2006. Angiogenesis Annu. Rev. Med. 57, 1–18.
Gravina, H.D., Antonelli, L., Gazzinelli, R.T., Ropert, C., 2013. Differential use of TLR2 and TLR9 in the regulation of immune responses during the infection with *Trypanosoma cruzi*. PLoS One 8, e63100.
Hu, D.E., Hiley, C.R., Smither, R.L., Gresham, G.A., Fan, T.P., 1995. Correlation of 133Xe clearance, blood flow and histology in the rat sponge model for angiogenesis: further studies with angiogenic modifiers. Lab. Investig. 72, 601–610.
Kim, Y.W., West, X.Z., Byzova, T.V., 2013. Inflammation and oxidative stress in angiogenesis and vascular disease. J. Mol. Med. 91, 323–328.
Koberle, F., 1961. Patologia y anatomia patológica de la enfermedad de Chagas. Bol. Oficina Sanit. Panam. 51, 404–428.
Lima, L.D.C., Andrade, S.P., Campos, P.P., Barcelos, L.S., Soriani, F.M., Moura, S.A.L., Ferreira, M.A.N.D., 2014. Brazilian green própolis modulates inflammation, angiogenesis and fibrogenesis in intraperitoneal implant in mice. BMC Complement. Alternat. Med. 14, 177–186.
López, N.C., Valck, C., Ramírez, G., Rodríguez, M., Ribeiro, C., Orellana, J., Maldonado, I., Albini, A., Anacona, D., Lemus, D., Aguilar, L., Schwaeble, W., Ferreira, A., 2010. Antiangiogenic and antitumor effects of *Trypanosoma cruzi* calreticulin. PLoS Negl. Trop. Dis. 6, e730.
Marques, S.M., Campos, P.P., Castro, P.R., Cardoso, C.C., Ferreira, M.A., Andrade, S.P., 2011. Genetic background determines mouse strain differences in inflammatory angiogenesis. Microvasc. Res. 82, 246–252.
Molina, M.C., Ferreira, V., Valck, C., Aguilar, L., Orellana, J., Rojas, A., Ramirez, G., Billetta, R., Schwaeble, W., Lemus, D., Ferreira, A., 2005. An *in vivo* role for *Trypanosoma cruzi* calreticulin in antiangiogenesis. Mol. Biochem. Parasitol. 140, 133–140.
Moura, S., Negri, G., Salatino, A., Lima, L.D., Dourado, L.P., Mendes, J.B., Andrade, S.P., Ferreira, M.A., Cara, D.C., 2009. Aqueous extract of Brazilian green propolis: primary components, evaluation of inflammation and wound healing by using subcutaneous implanted sponges. Evid. Based Complement. Alternat. Med. 2011, 748283–748291.
Ploeger, D.T., van Putten, S.M., Koets, J.A., van Luyn, M.J., Harmsen, M.C., 2012. Human macrophages primed with angiogenic factors show dynamic plasticity, irrespective of extracellular matrix components. Immunobiology 217, 299–306.
Prata, A., 2001. Clinical and epidemiological aspects of Chagas disease. Lancet Infect. Dis. 1, 92–100.
Ramírez, G., Valck, C., Ferreira, V.P., López, N., Ferreira, A., 2011. Extracellular *Trypanosoma cruzi* calreticulin in the host–parasite interplay. Trends Parasitol. 27, 115–122.
Ramírez, G., Valck, C., Aguilar, L., Kemmerling, U., López-Muñoz, R., Cabrera, G., Morello, A., Ferreira, J., Maya, J.D., Galanti, N., Ferreira, A., 2012. Roles of *Trypanosoma cruzi* calreticulin in parasite–host interactions and in tumor growth. Mol. Immunol. 52, 133–140.

Rassi Jr., A., Rassi, A., Marin-Neto, J.A., 2009. Chagas heart disease: pathophysiologic mechanisms, prognostic factors and risk stratification. Mem. Inst. Oswaldo Cruz 104 (Suppl. 1), 152–158.
Talvani, A., Teixeira, M.M., 2011. Inflammation and Chagas disease: some mechanisms and relevance. Adv Parasitol 76, 171–194.
Teixeira, A.S., Araújo, F.A., Ferreira, M.A., Barcelos, L.S., Teixeira, M.M., Andrade, S.P., 2005. Angiogenesis and inflammation in skeletal muscle in response to ascites tumor in mice. Life Sci. 78, 1637–1645.